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Press

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is a summary of the following publications, which are referred to in the text by their Roman numerals. All published articles are reprinted here with permission from the copyright owners.

I. **Hindrikson, M.**, Männil, P., Ozolins, J., Krzywinski, A, Saarma, U. (2012) Bucking the Trend in Wolf-Dog Hybridization: First Evidence from Europe of Hybridization between Female Dogs and Male Wolves. PLoS ONE 7(10): e46465.

II. **Hindrikson, M.**, Remm, J., Männil, P., Ozolins, J., Tammeleht, E., Saarma, U. (2013) Spatial Genetic Analyses Reveal Cryptic Population Structure and Migration Patterns in a Continuously Harvested Grey Wolf (*Canis lupus*) Population in North-Eastern Europe. PLoS ONE 8(9): e75765.

III. **Hindrikson, M.**, Remm, J., Pilot, M., Godinho, R., Stronen, A.V., Baltrūnaité, L., Czarnomska, S.D., Leonard, J.A., Randi, E., Nowak, C., Åkesson, M., López-Bao, J.V., Álvares, F., Llaneza, L., Echegaray, J., Vilà, C., Ozolins, J., Rungis, D., Aspi, J., Paule, L., Skrbinšek, T., Saarma, U. (2016) Wolf population genetics in Europe: a systematic review, meta-analysis and suggestions for conservation and management. Biological Reviews, doi: 10.1111/brv.12298.

My personal contribution to the articles referred to in the thesis is as follows:

I–III – responsible for laboratory procedures, participation in data analyses and writing the first draft of the articles.

1. INTRODUCTION

The grey wolf *Canis lupus* L. 1758 was historically widespread across Europe, however, the species was progressively eradicated from most of the continent between the 18th and 20th centuries (Chapron *et al*., 2014; De Groot *et al*., 2016). Once viewed primarily as a threat to public safety and livestock, but also as a competitor on wild game, the grey wolf (henceforth 'wolf') has recently become recognized as an apex predator that plays a key role in ecosystems (Bruskotter, Enzler & Treves, 2011). Ongoing protection of European wilderness zones, socio-economic changes, innovative laws, public and political commitment, recovery of wild ungulate species and wolf dispersal ability have enabled the species to recolonize many parts of its former range in Europe (Boitani, 1992; Musiani, Boitani & Paquet, 2009, 2010; Randi, 2011; Chapron *et al*., 2014; Leonard, 2014; Gilroy, Ordiz & Bischof, 2015; López-Bao *et al*., 2015). Currently, ca 12 000 wolves occupy over 800 000 square kilometres in 28 European countries, with 9 900 individuals present in 22 countries belonging to the European Union (Chapron *et al*., 2014).

Anthropogenic activities are one of the key factors influencing wildlife populations, and probably the most important among them are overexploitation and habitat destruction or fragmentation, which cause a considerable range of problems for wildlife (Allendorf & Luikart, 2007). However, despite recolonization processes going on in a number of European wolf populations, there are populations that have dramatically decreased in size and have become increasingly fragmented. In addition to reduction in population size and density, which are usually considered, severe hunting pressure can lead also to population fragmentation, increased immigration from other populations, disruption of social systems (e.g. Allendorf *et al*., 2008) and can increase the potential for hybridization between wild and closely related domestic animals, which is especially relevant for domesticated dogs and wild canids.

Wolf has long been a subject of scientific curiosity. In recent decades, application of genetic methods has greatly broadened our understanding of processes underlying wolf population structure, dynamics, phylogeography, hybridization with other canids (especially dogs) and domestication, to name the most important.

1.1. Genetic markers used in European wolf populations

Six main types of genetic markers have been used to study wolves: (1) autosomal microsatellites, (2) autosomal single nucleotide polymorphisms (SNPs), (3) major histocompatibility complex (MHC), (4) mitochondrial DNA (mtDNA), (5) Y chromosome microsatellites and (6) Y – SNPs (Fig.´s 2, 3 in **III**; note that marker types 3 and 6 are not shown in figures). While mtDNA was initially the

most frequent choice, autosomal microsatellites quickly gained popularity due to their superior power of identifying individual animals and for assessing population genetic diversity, population structuring and rates of gene flow between populations. Recently, the depth of population analysis has been increased further by large-scale genome-wide SNP data (vonHoldt *et al*., 2011; Stronen *et al*., 2013; Pilot *et al*., 2014*b*). As a general rule in mammals, mtDNA is maternally inherited and cannot alone represent all historical and contemporary processes acting upon populations. Nuclear data derived from biparental autosomal loci or from the paternal Y chromosome are thus required to gain more complete understanding of evolutionary and contemporary population processes of wolves across Europe. Contrary to mtDNA and SNP data that can be combined between studies to cover large areas, the use of microsatellites has an important shortcoming: the data cannot be easily compared between different studies (De Groot *et al*., 2016). Thus, some microsatellite data sets have been used for specific countries or for a limited region (Flagstad *et al*., 2003; Jędrzejewski *et al*., 2005; Fabbri *et al*., 2007; Jansson *et al*., 2012, 2014; Moura *et al*., 2014), with few used to characterize wolf populations from wider areas (Pilot *et al*., 2006, 2014*a*; Aspi *et al*., 2009; Sastre *et al*., 2011; Fabbri *et al*., 2014). The genetic diversity of wolf populations which have suffered demographic bottlenecks and recoveries has also been investigated at the level of loci encoding proteins for the major histocompatibility complex (Seddon & Ellegren, 2004; Arbanasić *et al*., 2013; Galaverni *et al*., 2013; Niskanen *et al*., 2014). In addition to the maternal and biparental markers, investigations of the paternal lineage (Y chromosome) have become also increasingly frequent, though the majority have focused primarily on wolf-dog hybridization (Sundqvist *et al*., 2001; Vilà *et al*., 2003*b*; Iacolina *et al*., 2010; Godinho *et al*., 2011; Fabbri *et al*., 2014; Randi *et al*., 2014).

1.2. Population structure

Grey wolves are capable of adapting to a wide range of ecological conditions. Recent evidence suggests that the social organisation of wolves into packs might be one of the reasons explaining the evolutionary success of the species; packs enable wolves to effectively use a wide range of resources to feed and guarantee better survival of their young (Stahler *et al*., 2006; Sand *et al*., 2006; vonHoldt *et al*., 2008). Under natural conditions, i.e. in the absence of strong hunting pressure, wolves generally live in kin-based packs containing a dominant pair of adults, their offspring and close relatives (Mech & Boitani, 2007; Jędrzejewski *et al*., 2005). Packs are usually nomadic within territories (Mech & Boitani, 2007). When the offspring mature, they mostly disperse and live solitarily for a period before finding a mate and territory and producing offspring (Mech & Boitani, 2007) but vonHoldt *et al*. (2008) has described several other mechanisms for finding a mate. Severe hunting pressure can, however, break up this natural social structure into smaller entities (Valdmann, Laanetu &

Korsten, 2004; Moura *et al.*, 2014) with the adoption of unrelated individuals into packs, resulting in low kinship (Jędrzejewski *et al*., 2005; Rutledge *et al*., 2010) and sometimes territory abandonment (Brainerd *et al*., 2008) and hybridization with dogs (Vilà & Wayne, 1999; Andersone *et al*., 2002; Randi, 2008). Although it has been proposed that wolf populations compensate for human exploitation via adjustments in dispersal, including immigration (Jędrzejewska *et al*., 2006; Adams *et al*., 2008), a strong association has been found between human offtake and total mortality rates of wolves in North-America (Creel & Rotella, 2010).

The need for genetic studies of Estonian and Latvian wolves became urgent when in short time period (2008–2009) altogether six wolves with unusual coloration (black and yellow) were shot by hunters and suspected as putative hybrids. Rapid development of several genetic methods in wolf population studies (Vilà *et al*., 1997; Randi & Lucchini, 2002; Vilà *et al*., 2003*b*; Fabbri *et al*., 2007; Pilot *et al*., 2006, 2010) made approaches using genetic material most appropriate to confirm the hybrid status of these animals. Until this point, mainly ecology of wolves had been studied in Estonia and Latvia, including wolf diet (Kübarsepp & Valdmann, 2003; Andersone & Ozolins, 2004; Valdmann *et al*., 2005), prey selectivity (Valdmann, Koppa & Looga, 1998) or parasites (Moks *et al*., 2006). Besides identification of hybridization in the Estonian and Latvian wolves, genetic methods would be most appropriate also for studying population structure, connectivity with neighbouring populations and spatial patterns that were unknown in this part of Baltic wolf population.

Wolf populations in Estonia and Latvia are believed to be part of the continuous Baltic wolf population which extends through all three Baltic countries Estonia (200−260 individuals), Latvia (200–400) and Lithuania (300–400) and northeastern Poland (270−360), comprising 900−1400 animals in total (**III** and **Fig. 1**). The Baltic population is connected to wolf populations in eastern Poland (Czarnomska *et al*., 2013), western continental Russia, northern Ukraine and Belarus (Boitani & Ciucci, 2009). As in other parts of Europe, the Baltic wolf population experienced near-extermination in the 1970s and 1980s (Jędrzejewski et al., 2005; Baltrūnaitė, Balčiauskas & Åkesson, 2013) leaving signs of genetic bottlenecks in wolves from Estonia (Plumer *et al*., 2016) and neighbouring Russia (Sastre *et al*., 2011). Estonian and Latvian populations, to be specific, went through severe decline around the mid-1960s, when the estimated average population size was in some years as low as 13 individuals in Latvia and nine in Estonia. Populations in both countries started to recover in the second half of the 1970s and reached their maximum in the middle of 1990s, when in Estonia and Latvia the population census sizes were about 700 and 900–1000 animals, respectively. During that period, hunting pressure also escalated, with annual harvests constituting from one third to nearly half of the population census in both countries. Most probably as a result of the severe hunting pressure putative wolf-dog hybrids started to appear in both countries, and the hybrid status of several individuals has been verified with genetic analysis in Latvia (Andersone *et al*., 2002).

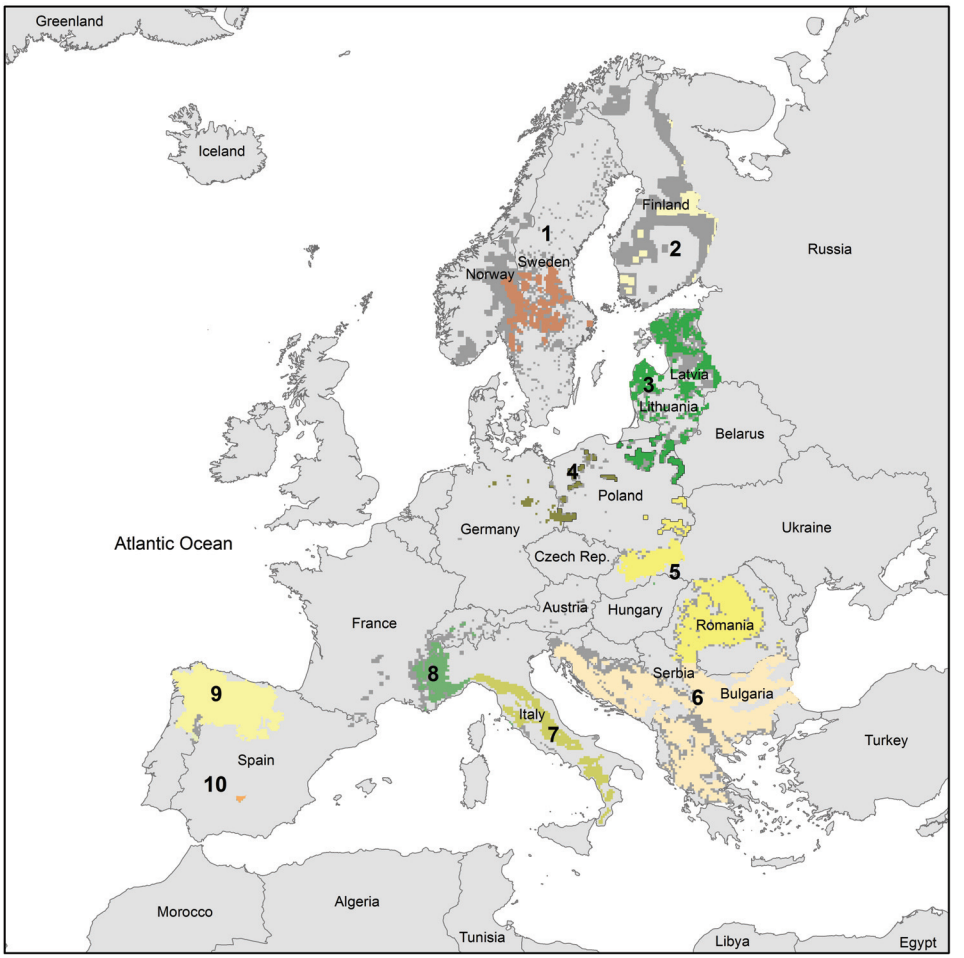

Figure 1. Wolf populations in Europe according to Chapron *et al.* (2014). Different colours indicate wolf permanent occurrence and dark grey sporadic occurrence (modified from Chapron *et al.,* 2014). Wolf occurrence in Russia, Ukraine and Belarus is not marked on the map. 1 – Scandinavia; 2 – Karelia; 3 – Baltic; 4 – Central European Lowlands; 5 – Carpathian; 6 – Dinaric-Balkan; 7 – Italian peninsular; 8 − Alpine; 9 – North-west Iberia; 10 – Sierra Morena.

In general, the Baltic population exhibits relatively high levels of heterozygosity compared with many other European wolf populations (Jędrzejewski *et al*., 2005; Baltrūnaitė, Balčiauskas & Åkesson, 2013; Czarnomska *et al*., 2013). Currently, Estonian wolf population is expanding and has recently (presumably in 2010–2011) re-colonized the two largest islands of the country, Saaremaa and Hiiumaa (Plumer *et al*., 2016).

Detecting genetic structure is of major importance for the effective conservation and management of wolf in multiple scales from local population inside one country borders to biologically determined populations such as Baltic

population to across European scale including altogether ten wolf populations in Europe. However, while population structuring on a local populational scale provide important information on processes of population dynamics and demographic history (Aspi *et al*., 2006), genetic viability (Jansson *et al*., 2012, 2014), inbreeding (Liberg *et al*., 2005), large-scale patterns such as genetic consequences of glaciations (Pilot *et al*., 2010), population connectivity and admixture (Stronen *et al*., 2013), mechanisms of gene flow, long-time population isolations or range expansions (Pilot *et al.,* 2014*b*) or ecological factors influencing population structure (Pilot *et al*., 2006) are adequately revealed in studies including wolves all over Europe. However, so far, main studies on wolf population structure have been performed on local populations (Jędrezejewski *et al*., 2005; Liberg *et al*., 2005; Aspi *et al*., 2006, 2009; Fabbri *et al.*, 2007; Jansson *et al*., 2012, 2014; Baltrūnaitė, Balčiauskas & Åkesson, 2013; Moura *et al*., 2014; Karamanlidis *et al*., 2016), while studies on a wider scale and data from across Europe have been lacking until recent years (Pilot *et al*., 2006, 2010, 2014*b*; Stronen *et al*., 2013). While De Groot *et al*. (2016) provided a review of genetic markers used and emphasized a harmonization of methods, publication **III** represents an analytical summary of the main findings from wolf population genetic studies in Europe, covering major studies from the 'pre-genomic era' and the first insights of the 'genomics era'.

1.3. Wolf-dog hybridization

There is growing evidence that many animal species can hybridize: Mallet (2005) has proposed that at least 6% of European mammal species undergo some degree of hybridization. Usually, the population level impact of hybridization is believed to be minor; however, where introgression occurs, a substantial number of maladapted genes or alleles can enter parental populations (Leonard *et al*., 2014). A classic example of the conservation implications of this process is the hybridization between wolves and its domestic form, the dog. Phylogenetic studies place dogs and wolves as sister taxa (Lindblad-Toh *et al.,* 2005; Wayne & Ostrander, 2007). Dogs were the first domesticated species, originating at least 15 000 years ago from Eurasian grey wolves (Shannon *et al*., 2015), but simple scenarios of dog domestication are confounded by extensive admixture (Zhenxin *et al*., 2016). Moreover, no single wolf population is more closely related to dogs, supporting the hypothesis that dogs were derived from an extinct wolf population (Zhenxin *et al*., 2016).

As grey wolves and domestic dogs possess identical karyotypes and can hybridize to produce fertile offspring in the wild (Vilà & Wayne, 1999), the main conservation concern is the significant reduction or loss of specific adaptations that could lead to the extinction of already small and fragmented wolf populations if introgression is sufficiently frequent. Hybridization may be followed by introgression, a process through which maladapted genes may enter in wild populations: the gene pool of each species is the result of thousands to millions

of years of selection and adaptation to specific environmental conditions and a rapid influx of genes from another species, that evolved under different conditions can result in a depression of fitness (Leonard *et al*., 2014). Such genes could reduce the population's chances of long-term survival and in case of high frequency of hybridization and introgression, a major conservation threat is the potential for the loss of the genetic integrity of a species (Caniglia *et al*., 2013; Leonard *et al*., 2014).

Grey wolves are not the only wild species that can hybridize with dogs. All wolf-like canids (*Canis* spp. and *Lycaon)* have the same number of chromosomes (n = 78) (Leonard *et al*., 2014) and may have the capacity to interbreed and produce viable offspring (Vilà & Wayne, 1999), indicating incomplete reproductive isolation. Moreover, hybridization coupled with subsequent introgression is a documented threat to a number of canids, including the the red wolf (*C. rufus*) (Wayne and Jenks, 1991; Adams, Kelly & Waits, 2003), Ethiopian wolf (*Canis simensis*) (Gotelli *et al*., 1994) and the dingo (*C. lupus dingo*) (Elledge *et al*., 2008; Stephens *et al*., 2015). Although hybridization can affect wild canids in a number of ways, the negative impacts are better known, including loss of reproductive potential or genetic integrity of a population, lowered fitness of hybrid individuals or introduction of maladaptive alleles into wild populations (Leonard *et al*., 2014).

Although there are great examples of European wolf populations that are increasing their area, there are populations that have dramatically decreased in size and have become fragmented (like some parts of Iberian wolf population, MAGRAMA, 2016) or isolated (Scandinavian population, Seddon *et al*., 2006; Laikre *et al*., 2013), mainly due to extensive hunting pressure and habitat loss during recent centuries. Remaining populations are exposed to increasing numbers of humans and dogs (Boitani, 2003). In Europe, there is genetic evidence of hybridization from Scandinavian (Vilà *et al*., 2003*b*), Baltic (Andersone *et al*., 2002; Stronen *et al*., 2013), Dinaric-Balkan (Randi *et al*., 2000; Vilà *et al.*, 1997; Stronen *et al*., 2013; Moura *et al.,* 2014; Majić-Skrbinšek, 2014), Italian peninsular and Alpine populations (Randi *et al.,* 2000; Randi & Lucchini, 2002; Verardi, Lucchini & Randi, 2006; Iacolina *et al.,* 2010; Caniglia *et al.,* 2013; Boggiano *et al.,* 2013; Randi *et al.,* 2014; Lorenzini *et al.,* 2014) and in Iberian population (Godinho *et al.,* 2011, 2014). This evidence has most often been based on mtDNA (Vilà *et al.,* 1997; Randi *et al.,* 2000), autosomal microsatellite variation (Randi *et al.,* 2000; Randi and Lucchini, 2002; Verardi, Lucchini & Randi, 2006; Lorenzini *et al.,* 2014; Godinho *et al.,* 2014) or in combination of these markers (Boggiano *et al.,* 2013; Moura *et al.,* 2014) with only small number of studies additionally using Y chromosome data (Vilà *et al.,* 2003*b*; Ciucci *et al.*, 2003; Iacolina *et al.,* 2010; Godinho *et al*., 2011; Caniglia *et al.,* 2013) to investigate the role of both genders in the hybridization process.

1.4. The objectives of this thesis

Publications **I** and **II** provide previously unknown information about wolves in Estonia and Latvia, for there has been no study that has looked into the fine scale wolf genetic structure of any Baltic country before. Although Estonian and Latvian wolves show high levels of genetic variation (**I** and **II**) and the number of wolf packs has stayed between 15 to 31 during last 10 years, this part of the Baltic population faces pressing conservation threats. The recent decreasing trend in census size together with increasing pressure within local communities due to predation on domestic animals (Plumer *et al*., 2016), identification of wolf-dog hybrids and arrival of golden jackal (*C. aureus*) in Estonia warrants a more detailed and science-based wolf management practices. This is particularly relevant within the context of population connectivity inside Baltic population because uncoordinated management in neighbouring countries facing similar problems may result in harmful effects on population-level genetic viability and health.

The main objectives of this thesis were: (1) to analyse hybridization between wolves and dogs in Estonia and Latvia; (2) to use novel spatial genetic approaches to analyse population structure and patterns of gene flow in the wolf population in Estonia and Latvia, and (3) to analyse general large-scale trends and patterns of genetic variation in European wolf populations, and to provide comprehensive overview of wolf population processes in Europe, based on the results derived from three lineages −maternal (mtDNA), paternal (Y-chromosome microsatellites) and biparental (autosomal microsatellites and single nucleotide polymorphisms or SNPs) − in Europe.

The hypotheses were: (1) hybridization has occurred in Estonian and Latvian wolf population and the direction of these events has been between male wolves and female dogs; (2) Estonian-Latvian wolf population is panmictic and without genetic clustering; (3) general large-scale trends and patterns of genetic variation in European wolf populations reach beyond the country borders, involving multiple countries.

To achieve these aims, we used: (1) combined genetic analysis of mtDNA, biparental autosomal and Y chromosome-specific microsatellite loci for identification of wolf-dog hybrids (**I**); (2) two novel methodological approaches that further the analytical possibilities of the programme Structure, and spatially explicit individual-based method DResD (distribution of residual dissimilarity) to analyse structure and migrations in wolf population in Estonia and Latvia. We applied DResD for the first time to microsatellite data, revealing a migration corridor and barriers, and several contact zones (**II**); (3) results of previous microsatellite-based studies and also included new data (**III**) to describe general large-scale trends and patterns of genetic variation in European wolf populations, we analysed.

2. MATERIAL AND METHODS

2.1. Samples for DNA analysis

Muscle tissue samples of 266 grey wolves were collected across the species range in Estonia and Latvia between the 2001/2002 and 2009/2010 hunting seasons (**I–II**). 74 samples (**I**) and 116 samples (**II**) were used for microsatellite analysis. All samples were collected from animals legally harvested by hunters for other purposes than these studies, including the muscle tissue samples from eight shot putative wolf-dog hybrids (**I**). Blood samples analysed from dogs (21) were obtained from local veterinarians (**I)**. Samples were stored in 96% ethanol at –20 ˚C prior to extraction of genomic DNA. Total genomic DNA was extracted either from muscle tissue or blood using High Pure PCR Template Preparation Kit (Roche Diagnostics) following manufacturers´ protocols.

2.2. Genetic analyses

Microsatellite genotyping was performed on 11 (**I**) or 16 (**II**) autosomal loci and 7 Y-chromosome specific loci (**I**) using primer pairs previously described in the literature. Autosomal loci used were as follows: (1) for **I**: FH2001, FH2010, FH2054, FH2079, FH2088 (Francisco *et al.,* 1996), vWF (Shibuya *et al*., 1994), AHT130 (Holmes *et al*., 1995), M-CPH2, M-CPH12 (Fredholm and Winterø, 1995) and C20.253, CXX22 (Ostrander, Sprague & Rine, 1993); (2) for **II**: FH2001, FH2010, FH2017, FH2054, FH2079, FH2088, FH2096 (Francisco *et al.,* 1996), vWF (Shibuya *et al.,* 1994), AHT130 (Holmes *et al.,* 1995), M-CPH2, M-CPH4, M-CPH12 (Fredholm and Winterø, 1995) and C466, C09.173, C20.253, CXX22 (Ostrander, Sprague & Rine, 1993). Y choromosome specific loci used in **I** were as follows: MS34A, MS34B, MS41A, MS41B (Sundqvist *et al.,* 1991), 990–35, 650–79.2 and 650–79–3 (Bannasch *et al.,* 2005). For publication **I**, additionally 1673 bp of mitochondrial DNA control region (mtDNA CR) was sequenced, using newly developed primers. Reaction conditions and primer sequences can be found in Materials and Methods of **I–II**.

Amplified products for microsatellite analyses (**I–II**) were diluted in distilled water (5x dilution) and separated on an ABI 3130xl automated sequencer (Applied Biosystems) in three capillaries as described in **I–II**. Sequences of mtDNA were resolved on ABI PRISM 377 or 3130xl automated sequencers (Applied Biosystems). For fragment sizing in articles **I–II**, a GeneScan 500 LIZ size standard (Applied Biosystems) was used and microsatellite alleles were sized and genotyped with Gene Mapper v4.0 (Applied Biosystems).

2.3. Statistical analyses

2.3.1. Identification of wolf-dog hybrids

2.3.1.1. Analysis of autosomal and Y chromosome microsatellite loci

Biparental multilocus genotypes and Y-chromosome haplotypes in article **I** were analysed with:

A) Structure v2.2 (Pritchard, Stephens & Donnelly, 2000) was used to evaluate the number of genetic clusters (K) in the data and to assign individuals to their likely origin. For identification of hybrid samples, the dataset consisted of all 103 individuals, including wolves (n= 74), dogs (n =21) and eight hybrids from Estonia (n= 6) and Latvia (n= 2). The parameters used to run Structure can be found in Material and Methods section of article **I**. We estimated the number of clusters K as suggested by Evanno, Regnaut & Goudet (2005).

B) a Bayesian model-based clustering method for identifying hybrids was performed with Newhybrids v1.1 beta (Anderson & Thompson, 2002). The method identifies hybrid individuals on the basis of the posterior probability of belonging to different pure parental or hybrid categories generated during n = 2 or n = 3 generations of potential interbreeding. Four distinct genotype frequency classes were simulated using Hybridlab v1.0 (Nielsen, Bach & Kotlicki, 2006) on the basis of pure species I (Wolf) and pure species II (Dog): F1 wolf-dog hybrids (n= 100) and F2 hybrids (F1 hybrid x F1 hybrid; n= 100), including backcrosses with pure species (F1 hybrid x wolf; n= 100) and (F1 hybrid x dog; n= 100). Parameters for simulations can be found in the Material and Methods section of the article **I**. Sibling relationships and relatedness among hybrids was investigated using Kingroup v2.0 (Konovalov, Manning & Henshaw, 2004) and the relatedness estimator according to Konovalov & Heg (2008).

C) Factorial correspondence analysis (FCA) implemented in Genetix v4.05.2. (Belkhir *et al*., 2004) was used to distinguish wolves, dogs and wolf-dog hybrids on the basis of microsatellite data.

D) Based on microsatellite data from the Y-chromosome loci of wolves, dogs and hybrids (n = 45), a median joining network was calculated with the program Network v4.510 (Bandelt, Forster & Röhl, 1999) in **I**.

2.3.1.2. Mitochondrial DNA analysis

A minimum spanning network was calculated with Network v4.510 using a median-joining approach (Bandelt, Forster & Röhl, 1999) in publications **I** and **III.** The network in publication **I** was based on partial mtDNA control region (the final length after alignment and trimming was 1134 bp) and included wolves, dogs and hybrids from Estonia and Latvia (**I**, Table S3). For further analysis of phylogenetic relationships between hybrids, wolves and dogs, the dataset was

expanded by including additional 95 dog and eight wolf homologous 1134 bp mitochondrial control region data from GenBank (only those that had complete 1134 bp sequence available without ambiguous sites) (Arnason *et al*., 2007; Bjornerfeldt, Webster & Vilà, 2006; Kim *et al*., 1998; Webb & Allard, 2009), thus, in the final analyses 213 sequences were used (**I**; Table S3). In publication **III**, 160 publicly available 609 bp mt DNA control region sequences covering all wolf populations in Europe and geographically close populations in West Asia.

In publication **I**, the appropriate model of sequence evolution was calculated with jModeltest v1.0.1 using the Bayesian Information Criterion (Posada, 2008). Phylogenetic trees were generated using Bayesian inference (BI) implemented in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). The parameters used for MrBayes can be found in Material and Methods section of article **I**. To ensure that the BI was not trapped in local optima, the analysis was performed three times. Phylogenetic trees were visualized with FigTree v1.3. (http://tree.bio.ed.ac.uk/ software/figtree).

2.3.2. Population structure analysis

In publication **II**: Bayesian assignment tests were performed with Structure v2.2 (Pritchard, Stephens & Donnelly, 2000) to evaluate the number of genetic clusters (*K*) and to assign individuals to their likely origin. Assignment of individuals into genetic clusters was performed with Structure (methodology was identical with the ones in **I**). FCA implemented in Genetix v4.05.2 (Belkhir *et al*., 2004) was additionally used to investigate population sub-structuring.

The data set was tested for IBD (isolation-by-distance) as shown in the Material and Methods of article **II** in this dissertation. For estimating the ranges of the genetic groups, grid points were classified according to three alternative hypotheses (see Materials and Methods in Meta-analysis in **III**). To identify potential regions of the study area that might represent corridors or barriers to migration, as well as core, transition and blending areas of population subgroups, we performed DResD analysis, which is a recently introduced spatially explicit, individual-based approach that is based on IBD modelling and pairwise geographic and genetic distances (Keis *et al*., 2013). For the DResD procedure applied in this study, see **II** (Information S1 for the full script in R 2.14 language).

2.3.3. Meta-analysis of European populations

To describe general large-scale trends and patterns of genetic variation in European wolf populations, we analysed the results of previous microsatellite studies and included new data, which altogether covered nine European wolf populations in 19 countries: Russia, Norway, Sweden, Finland, Estonia, Latvia,

Lithuania, Poland, Germany, Czech Republic, Slovakia, Belarus, Italy, Croatia, Bulgaria, Bosnia and Herzegovina, Greece, Spain and Portugal (Appendix S4; Fig. 5 in **III**) (note that the Sierra-Morena population is missing due to a lack of data, while data for Russian populations are included). Observed and expected heterozygosities (H_O, H_E), inbreeding coefficient (F_{IS}), allelic richness (R_A) that uses a rarefaction on the minimum number of samples per populations in the study, and the number of alleles per locus (N_A), were extracted.

Linear trend surface analysis was applied to each variable to determine the presence and direction of a gradient (Fortin & Dale, 2005), followed by a test of the spatial trend. The analysis calculated spatial autocorrelation (SAC) structure via variogram modelling and spatial weighting. The program R function *gls* with spherical SAC structure in the package *nlme* (Pinheiro *et al*., 2013) with rotated geographic coordinates along the gradient direction. The details for determing the constant azimuthal direction can be found in the Materials and Methods section of the publication **III**. Due to a relatively small number of data-points, we focused on general patterns and did not test non-linear effects, but analysed the pattern in the ten European populations separately. After the trend surface analysis, the presence of residual spatial autocorrelation was tested using Moran's autocorrelation index (*I*) and the compatible test of significance in the R package *ape* (Paradis, Claude & Strimmer, 2004).

3. RESULTS

3.1. Detecting wolf-dog hybridization in Estonia and Latvia (I)

Based on allele frequencies at 11 autosomal microsatellite loci, (1) eight wolf-dog hybrids carried several alleles that were also found in wolves but were absent in dogs or vice-versa and (2) clear distinction of wolves, dogs and hybrid animals from each other in the FCA analysis appeared. The wolf-dog hybrids from Estonia and Latvia were assigned into two clusters according to their geographic location, and all eight hybrids were placed between dogs and wolves.

Assignment tests were carried out to determine whether the eight hybrids differed significantly from wolves and dogs in Estonia and Latvia. In different runs (with allele frequencies correlated or independent) used to identify putative hybrids, the number of genetic clusters estimated was always two (K = 2). With 'parameter set A', hybrids from Estonia were assigned with somewhat higher values to the wolf cluster, whereas hybrids from Latvia had similar probability values of belonging to wolf and dog clusters. However, with 'parameter set B', all eight hybrids from Estonia and Latvia were assigned with similar probability values to both wolf and dog clusters. Estonian-Latvian wolves exhibited membership coefficients ($q \geq 0.72$ (90% credible regions 0.62–1.00) of belonging to the wolf cluster.

According to the kinship analysis, some of the Estonian hybrids were full siblings, while others were more distantly related. Therefore, it is possible that Estonian hybrids were not descendants of same parents. The Latvian hybrids were full siblings.

Matrilineal phylogenies based on mtDNA control region haplotypes showed, that wolves and dogs were clearly divided into two distinct haplogroups. All six hybrids from Estonia carried sequences identical to the major wolf haplotype, while the two hybrids from Latvia shared a unique haplotype and grouped together with dogs. The larger phylogeny (included homologous wolf and dog sequences from GenBank) revealed two large clades: one specific to dogs and another that included both wolves and dogs. Both hybrids from Latvia were positioned in the dog-specific clade, while the six hybrids from Estonia were positioned in the wolf-dog clade. Network analysis of Y chromosome microsatellite loci demonstrated that both hybrid male individuals were more closely related to dog than wolf haplotypes, suggesting that their paternal lineage was most likely of dog origin.

3.2. Detection of cryptic population structure in Estonian and Latvian wolf population (II)

Allele frequency distributions revealed some evidence of recent population bottlenecks in the Estonian-Latvian wolf population: in the sign test conducted on all 16 microsatellite loci, the signatures of bottleneck were detected with the stepwise mutation model (SMM) and two phase model (TPM) models: wolf populations were not at mutation-drift equilibrium under SMM ($P < 0.0001$), with 16 loci out of 16 exhibiting heterozygosity deficiency; mutation-drift equilibrium was also not identified under TPM ($P = 0.006$; 12 loci with heterozygosity deficiency). Bottleneck was not statistically supported under the infinite alleles model (IAM) model ($P = 0.045$).

Bayesian clustering analysis using program Structure v2.3.4 (Pritchard, Stephens & Donnelly, 2000) (with IBD effect explaining only 6% of the variation), suggested the existence of four different genetic groups A-D (Fig. 2, Fig. S2 in **II**) with all genetic groups comprised individuals with a high average estimated membership coefficient for the respective group (Table 1 in **II**). The structuring of the Estonian-Latvian wolf population into distinct genetic groups gained further support from FCA analysis (Fig. S4 in **II**) and from the linear interpolation approach, which clearly identified the geographical ranges of the groups (Fig.´s 3, 4 in **II**). According to the range of core (*Hyp1A*) grid points, three of the four genetic groups were geographically well defined: groups A (covering 12.3% of the analysed land area) and D (covering 7.9% in two separate core areas) were Estonian-based, whereas group B (26.3% coverage) was Latvian-based. However, group C was distributed throughout Estonia and Latvia, with almost all land area falling within the probable range (*Hyp0*) of the group (and a core area with only 0.1% coverage). The credible range of group B (*Hyp1B* range) also included the majority of Estonia, while western Latvia was outside the credible ranges of groups B and D (Fig.´s 3, 4 in **II**). The DResD algorithm provided clear evidence of spatial variation of genetic divergence that is likely related to varying landscape resistance to individual movements. At each of the three spatial scales analysed, several areas appeared where the interpolated pairwise genotype likelihood ratio distance (D_{LR}) value was significantly higher or lower than expected from IBD alone (Fig. 5 in **II**). At the smallest spatial scale (20–80 km; Fig. 5a in **II**) several blending areas of different groups appeared with relatively high genetic distance between otherwise geographically closely positioned individuals. At the medium scale (80–140 km) a putative territory of an expanding pack was detected in the forested area in south-west Estonia, coinciding with one of the core areas of group D (Fig. 5b in **II**). At the largest spatial scale (140–250 km) a large area in the north-eastern part of Estonia was identified as a migration corridor, where individuals are genetically relatively similar over the large geographic distance. Moreover, the Gulf of Riga coincided with strong divergence between individuals (Fig. 5c in **II**).

3.3 Genetic variation and trends in European wolf populations (III)

We compared four indices of genetic diversity for ten wolf populations in Europe (Table 1; Fig. 5; Appendix S4 in **III**). Averaged genetic diversity was lowest in the populations in Iberia and Italy. The Iberian population was also characterised by the lowest allelic richness. The highest heterozygosity was observed in the largest population (Dinaric-Balkan, see Table 1 in **III**).

None of the variables were correlated with the size of the distribution area of analysed populations. The sample size was negatively correlated with two of the genetic indices (H_E: $R^2 = 0.32$, $p = 0.025$; R_A: $R^2 = 0.31$, $p = 0.03$). However, the observed heterozygosity and inbreeding coefficient had no correlation with sample size (H_O: $R^2 < 0.01$, $p = 0.71$; F_{IS}: $R^2 = 0.02$, $p = 0.70$). Sample size itself had no trend in Europe and was not affected by spatial autocorrelation.

There was a global spatial genetic trend of heterozygosities (H_O and H_E) in the European wolf population. Heterozygosity values were considerably higher towards the north-east and lower in south-western populations (Table 2a; Fig. 6 in **III**). The average range of connectedness of populations suggested that the mean size of wolf functional subunits is about 770 km, as indicated by the extent of significance of spatial autocorrelation on trend model residual values of H_O (650 km), H_E (800 km), and F_{IS} (850 km), (Table 2b in **III**). The reliability of the detected patterns was indicated by zero or near zero nugget effects of the variogram models. A small nugget effect indicates low variance among independent estimations (different studies) in the same geographic area, and, by extension, a robust pattern in the observed variable, and good repeatability of measured values. Allelic richness was distributed relatively evenly over Europe, having only a weak signal of spatial pattern and strong nugget effect of the variogram.

Various genetic diversity patterns were detected within the two large distinct regions of the wolf European range – Southern Europe (Iberia, Alps, Italy, Dinaric-Balkan), and North-East Europe (Russia, Karelia, Baltic, Carpathians, Central European Lowland). In the Scandinavian population, only limited data were available and it was not possible to include this region in the meta-analysis of regional patterns. In southern Europe, a significant gradient of H_O and H_E is directed toward the north-east (Table 3 in **III**). In the largest continuous population in north-east Europe, a significant west-east gradient of expected heterozygosity (H_E) was observed (Table 3 in **III**). The lowest values appeared in Germany and the highest in Estonia and Latvia (Fig. 6 in **III**). The trend model residuals of the H_E were not spatially autocorrelated (Moran's $I = 0.16$, $p = 0.088$). The observed heterozygosity (H_O), inbreeding coefficient (F_{IS}) and allelic richness (R_A) did not exhibit significant spatial trends in north-east Europe, and were significantly autocorrelated in space. The highest genetic variability (H_O and R_A) was found in Estonia and Latvia, and the lowest in Poland. Despite the relatively low heterozygosity in Germany, our results do not indicate significant inbreeding.

4. DISCUSSION

While some European populations such as Italian peninsular (Dolf *et al.*, 2000; Randi & Lucchini, 2002; Verardi, Lucchini & Randi, 2006; Fabbri *et al*., 2007, 2014; Lucchini, Galov & Randi, 2004; Scandura *et al*., 2011; Caniglia *et al*., 2013; Randi *et al*., 2014; Fabbri *et al*., 2014) and Scandinavian (Ellegren, Savolainen & Rosen, 1996; Ellegren, 1999; Flagstad *et al*., 2003; Vilà *et al*., 2003*a*, *b*; Seddon *et al*., 2005, 2006*;* Hagenblad *et al*., 2009) have been studied using genetic methods for number of years, the Baltic wolf population, and specifically what is concerning Estonian-Latvian wolves have received very little attention. Before publications **I** and **II** of this thesis, only Andersone *et al*. (2002) had investigated wolf-dog hybridization in Latvia using molecular methods. Although Estonian-Latvian wolves are forming only part of the larger Baltic wolf population (Chapron *et al*., 2014), the main results of our studies illustrate outstanding population processes occurring at a smaller spatial scale:

1. Hybridization between wolves and dogs. The first confirmed hybridization event between grey wolves and dogs in Estonia (in **I**) and the second time in Latvia: wolf-dog hybridization was previously reported in Latvia in 1999 and subsequently verified using genetic analysis (Andersone *et al*., 2002);
2. A rare case of direction of hybridization was identified in Latvian hybrids (male wolf x female dog) (**I**);
3. The Estonian-Latvian wolf population is characterised by relatively high genetic diversity (**II**) despite past bottlenecks and severe hunting pressure.
4. Our population analysis demonstrated that this relatively small wolf population shared between Estonia and Latvia is represented by four genetic groups (**II**).

The appearance of wolf-dog hybrids in both countries can most likely be explained by the combined effect of two factors: severe and continuous hunting pressure on wolf populations, together with the abundance of stray dogs. Intensive hunting may have the dual effects of reducing wolf population density and destroying the structure of wolf packs (Valdmann, Laanetu & Korsten, 2004; Jędrzejewski *et al*., 2005). Where wolf density is low and stray dogs are present, the probability of wolf hybridization with stray dogs may be increased. Stray dogs have long been present in Estonia and Latvia, reflecting the common practice of dog-owners in rural areas to let their dogs roam freely.

Microsatellites have been particularly useful for detecting genetic admixture between wolves and dogs (Andersone *et al*., 2002; Randi & Lucchini *et al*., 2002; Vilà *et al*., 2003*b*; Verardi, Lucchini & Randi, 2006; Munoz-Fuentes *et al*., 2010; Godinho *et al*., 2011) and in the article **I** hybridization was ascertained through combined analysis of biparental genetic markers (autosomal STR), paternal (Y chromosome STR) and maternal (mtDNA). Wolf-dog hybrids exhibited several alleles that were shared with one of the putative parent species but not the other, the mixed origin of hybrids was also indicated by other

analyses (factorial correspondence analysis and Structure): the results suggested that hybrids from Latvia are most likely F1, whereas generation of hybrids from Estonia was unequivocal (F1 or F2). While autosomal microsatellite data allowed the presence of wolf-dog hybrids in Estonia and Latvia to be established, gender-specific genetic markers were used to evaluate the direction of hybridization. According to the network analysis based on Y chromosome microsatellite loci and mtDNA analysis, for hybrids collected in Estonia, hybridization took place according to the common pattern, i.e. between female wolf and male dog. On the other hand, mtDNA haplotypes found in two Latvian hybrids grouped with dogs, representing an extremely rare case of hybridization between a female dog and a male wolf. This result provides a rare example that violates the general rule of sexual asymmetry in mating between wolves and dogs and it is the first confirmed case from Europe to demonstrate that hybridization has occurred between female dog and male wolf. As the majority of microsatellite alleles were of wolf origin and the minority were shared with dogs, the most likely scenario to explain those data is that hybridization and subsequent introgression had taken place.

In Europe, introgressive hybridization has been suggested by a study of the Italian wolf population (Verardi, Lucchini & Randi, 2006) and in a recent study from the Iberian Peninsula (Godinho *et al*., 2011) and potentially in Estonian-Latvian wolf populations (**I**). In Iberian population, a minimum of 4% of the wolves showed signals of introgressive hybridization (Godinho *et al.,* 2011). Determining introgression in a wolf population is of critical importance because first-generation hybrids may backcross and bring maladapted genes in parental wolf population (Leonard *et al*., 2014).

Conservation and management of large carnivores is a substantial challenge for biodiversity conservation due to a strongly established hostility to these species, especially to wolf, in human history and culture (Chapron *et al*., 2014). Structuring of wild animal populations due to overexploitation and habitat degradation is of increasing conservation and management concern not only in Europe but worldwide. Therefore, estimating genetic variation and the degree to which populations are genetically structured is important for conservation planning. Estonian and Latvian wolves are characterised by relatively high genetic diversity (**I–II**; Plumer *et al*., 2016) despite past population bottlenecks and severe hunting pressure. However, the discovery of cryptic population structure in the Estonian and Latvian wolf population (publication **II**) was unexpected due to the high mobility that the species exhibits, the relatively small geographic area studied, and the lack of obvious movement barriers and ecological specialisation. Several processes might be expected to have promoted the emergence of distinct genetic groups following a bottleneck in the study area: (1) groups might be formed by immigrant individuals from different parts of Lithuania and Russia; or (2) groups might originate from spatially separated local wolf packs (with no significant immigration). Considering all the data, the most plausible scenario may be a combination of these two processes.

The wolf is one of the most mobile terrestrial mammal species, having the ability to disperse over long distances – Wabakken *et al*. (2007) have documented a dispersal distance of 1,092 km from southeast Norway to northeast Finland and a wolf originating from German-western Polish population was identified 800 km away in Denmark (Andersen *et al*., 2015). Thus, one might expect gene flow within Estonia and Latvia – a relatively limited geographic area (maximum extent 560 km) – to be considerable as there are no obvious movement barriers. Furthermore, the results of **III** suggested that the genetic diversity of a given wolf population can be influenced by populations up to 850 km away. The weak IBD effect observed in publication **II** suggests that wolves are capable of mixing all over the study area, suggesting that in the absence of hunting, observed groupings would probably merge. This has been shown in Italy, where the genetic clustering has been also detected at local scales, whereas it occurred due to other populational processes, namely high spatial stability of packs, combination of long-range dispersal, the preference to mate with unrelated individuals and a high young mortality rate (Scandura *et al*., 2011). Therefore, severe hunting pressure is likely the major factor behind (1) population substructuring (**II** and Plumer *et al*., 2016) and (2) identified hybridization in Estonian-Latvian wolves (in **I**). There are several reasons for this: (1) intensive hunting may have the dual effects of reducing wolf population density and destroying the structure of wolf packs (Valdmann, Laanetu & Korsten, 2004; Jędrzejewski *et al*., 2005). Low density and fragmented nature of wolf population coupled with the imbalance between a number of wolves and stray dogs can lead to increased probability of mating between wolves and dogs as shown in several cases (Verardi, Lucchini & Randi, 2006; Godinho *et al*., 2011; Moura *et al*., 2014). (2) Hunting has without doubt been the primary mechanism responsible for reducing the density of the Estonian-Latvian wolf population. Under severe hunting pressure, immigration rates are highly likely to increase due to appearance of vacant territories (Kojola *et al*., 2006) and hunting, through its effect on immigration rates, almost certainly remains the ultimate cause. In Finland (Aspi *et al*., 2006; Jansson *et al*., 2012, 2014) and Lithuania (Baltrūnaitė, Balčiauskas, Åkesson *et al*., 2013) where hunting pressure is low and immigration moderate or low, no population structuring has been observed. Therefore, on the basis of immigration alone, the much smaller territory of Estonia and Latvia might be expected to exhibit no population structuring at all. If significant immigration was ongoing, one might expect to detect genetic groups or clustering of individuals with relatively high genetic distance near border areas in Lithuania and Russia. The very same situation in wolf population has been described in Bulgaria, where hybridization between wolves and dogs, high genetic diversity and local population structure have been documented (Moura *et al*., 2014).

Low public acceptance due to livestock depredation, especially in Saaremaa and Hiiumaa islands in western Estonia (Plumer *et al*., 2016) and human-caused mortality, including illegal killing, are the biggest threats this part of Baltic wolf population is facing. However, large infrastructure developments and fragmentation of suitable habitats by intensive forestry can also pose a significant

threat. These problems are expected to remain, if not increased in the future, e.g. the forthcoming construction of Rail Baltic and new highways. Moreover, the new fence currently being built at the Estonian-Russian border will probably decrease gene flow between the wolf populations between the countries, though complete isolation is unlikely as wolves can cross Lake Peipus during winter-time. Although gene flow occurs between Latvia and Estonia (**II**), there is no information on the extent of gene flow and directionality in the whole Baltic population. Similarly, there is a lack of knowledge on gene flow with neighbouring populations (Central European Lowland population, Carpathian popuation, Karelian population and wolves in Western Russia).

In order to minimize hybridization between wolves and dogs, maintain high genetic diversity and gene flow, the most effective strategy would appear to be a long-term maintenance of wolf populations at stable densities and with the natural social structure preserved. However, despite high hunting pressure, the presence of stray dogs and hybridization, the results of publication **I** show that Estonian and Latvian wolves have largely remained genetically distinct from dogs, suggesting that introgressive hybridization in nature might be counteracted by selection or behavioural factors. Moreover, these wolves have maintained high genetic diversity and a degree of substructuring (in publication **II**).

The results of meta-analysis (**III**) confirmed the recorded population histories of wolves in Europe: during the period of demographic decline, larger populations survived in the Balkans and Eastern Europe, small and fragmented populations remained in the Iberian and Italian peninsulas, and the species was eradicated from central Europe and Scandinavia.

Historically, populations in southern Europe have been isolated for long periods of time, possibly for several thousands of years in the case of the Italian (Lucchini, Galov & Randi, 2004; Fabbri *et al*., 2007) and Iberian (Sastre *et al*., 2011) populations. Population decline, long-term geographical isolation and a lack of gene flow into the Italian and Iberian wolf populations explain the low genetic diversity and divergence from other European populations, indicated from microsatellite (Lucchini *et al*., 2004; Godinho *et al*. 2011; Sastre *et al*., 2011), mtDNA (Pilot *et al*., 2010) and SNP data (vonHoldt *et al*., 2011; Stronen *et al*., 2013; Pilot *et al*., 2014*b*). The most effective conservation strategy would require an increase in heterozygosity through elevated gene flow and population growth. The North-West Iberian population has been expanding naturally eastward and southward in Spain (Blanco *et al*., 1990), however in other areas it has disappeared (MAGRAMA, 2016), whereas in Portugal there are no signs of wolf population growth, especially in central Portugal, where the wolf may be on the verge of extinction (Boitani & Ciucci, 2009). Wolves from the Alpine population have reached the Iberian Peninsula in the last decade, but they currently remain in the Eastern Pyrenees and Catalonia, with no connectivity to the North-West Iberian wolf population (Valière *et al*., 2003; Lampreave *et al*., 2011; Sastre, 2011). Despite the low levels of genetic variability in Italian wolves, this population has active internal gene flow between subpopulations, in large part directed from the Apennines to the Alps (Fabbri *et al*., 2007). This

population has colonized the Alps, forming a new Alpine wolf population that is now coming in contact with wolves of Dinaric-Balkan origin in the east (Fabbri *et al*., 2014; Ražen *et al*., 2016), which may result in natural gene flow between the Alpine and Dinaric-Balkan populations in future.

The relatively high heterozygosity in north-eastern European populations (**III**) can largely be explained by their demographic connectivity to the large metapopulation in western Russia, which has long served as an important source of immigrants (Pilot *et al*., 2006, 2010; Boitani & Ciucci, 2009). Due to gene flow between different countries, the Baltic population shows medium to high levels of genetic diversity (Jędrzejewski *et al*., 2005; Czarnomska *et al*., 2013; **II**), despite strong hunting pressure (Jędrzejewski *et al*., 2005; **II**). However, despite the relatively high levels of heterozygosity, signs of inbreeding in north-eastern European wolf populations (Table 1 in **III**) was found. Signs of recent inbreeding have also been detected in eastern European wolf populations by Pilot *et al*. (2014*a*). Although inbreeding is naturally avoided in a wolf pack through wide variety of behavioural mechanisms (vonHoldt et al., 2008), it may increase under strong hunting pressure, which decreases population size and disrupts wolf social structure (Valdmann, Laanetu & Korsten, 2004; Jędrzejewski *et al*., 2005; Moura *et al*., 2014), potentially reducing the quality of traits that define apex predators (Ordiz, Bischof & Swenson, 2013).

Lower values of global spatial trends of heterozygosity in south-western populations (Table 2 in **III**) is probably the result of several factors: recent population demographic history (hunting pressure and bottlenecks), connectivity (isolation in peripheral areas of wolf distribution in Europe) and environmental variables. As environmental gradients in Europe have existed for a long period of time, they most likely have had an impact on genetic variability. The higher levels of heterozygosity in north-eastern Europe may be due to gene flow between northern and eastern European and Russian wolf populations (Aspi *et al*., 2009; Pilot *et al*., 2006). The range of spatial influence of 650−850 km (**III**) is very likely for it has been shown that wolves can disperse as far as 800 km and more (Wabakken *et al*., 2007; Andersen *et al*., 2015). However, gene flow can be restricted due to prey and habitat specialization (Pilot *et al*., 2006; Leonard, 2014) and human-built obstacles (Aspi *et al*., 2009). Radiotracking of wolves has also suggested that few individuals in northern Europe disperse more than 400 km (Kojola *et al*., 2009) – half of the genetic distance of spatial influence found in our results. Furthermore, few of the dispersal events contribute to gene flow because of human-caused mortality (Kojola *et al*., 2009; Liberg *et al*., 2012). These considerations should be taken into account in wildlife management plans dominated by anthropogenic landscapes to avoid significant drawbacks for smaller and more fragmented wolf populations (Delibes, 1990; **II**), particularly in southern regions (Randi, 2011).

Most of the ten European wolf populations face similar common threats: overharvesting (including poaching), low public acceptance and conflicts due to livestock depredation (Table 4; Fig. 8 in **III**), resulting most likely from a lack of knowledge and poor management structure, but also from livestock damage

and deep fears of wolf attacks on humans and dogs. Interactions with domestic dogs leading to disease transfer and/or hybridization have also generated concern (Leonard *et al*., 2014). However, other threats, such as habitat destruction and large fluctuations in prey base, are also relevant to the majority of populations. Thus, various human-related factors are undoubtedly the main source of threats to wolf populations in Europe, and the generally negative human attitude toward wolves has been and remains the primary threat to wolf populations. Historically, even infectious diseases (e.g. rabies, sarcoptic mange) have not had such a devastating impact on wolf numbers as negative human attitudes, resulting in severe hunting pressure (legal and illegal), which in many areas in Europe led to wolf eradication in the past and continues to threaten small endangered populations (e.g. in Sierra Morena). Hunting, as well as other direct anthropogenic threats can have genetic consequences (Allendorf *et al*., 2008), especially for social species such as the wolf (e.g. Creel & Rotella, 2010; Ausband *et al*., 2015).

The genetic-related threats affecting European wolf populations are: (1) restriction to gene flow that can result in considerable genetic drift and inbreeding. Severe reduction or loss of population connectivity inside and among European wolf populations is the most challenging factor that requires strong measures, especially in areas where hunting pressure on wolves has been strong for some time (Kaczensky *et al*., 2013; Jansson *et al*., 2014; Chapron & Treves, 2016; Plumer *et al*., 2016); (2) Wolf–dog hybridization is the second most common genetic-related threat in the majority of European wolf populations (Table 4 in **III**). Hybridization has been shown to increase under strong anthropogenic pressure, especially at the population periphery and in areas with high human-caused mortality (Andersone *et al*., 2002; Vilà *et al*., 2003*b*; Godinho *et al*., 2011; Leonard *et al*., 2014). Another factor that can play an important role in wolf–dog hybridization is the disruption of social structure due to high hunting pressure (Valdmann, Laanetu & Korsten, 2004; Jędrzejewski *et al*., 2005), that can potentially increase the risk of hybridization. Moreover, introgression (following hybridization) can bring selective genetic changes by introducing maladapted genes into wild populations (Leonard *et al*., 2014).

Large carnivores can coexist with humans if a favourable management policy is applied (Linnell, Salvatori & Boitani, 2008; Treves *et al*., 2016), however, recent recovery of wolf populations across Europe poses serious challenges to the management of the species.

SUMMARY

Knowledge of wolf population genetics and hybridization between wolves and dogs are of great importance for effective conservation and management of the species at both local and global scales. There are numerous studies published on wolf population genetics in different countries and regions in Europe, however the data on Baltic population, specifically part of the population represented by Estonian and Latvian wolves, were lacking. Studies including all European countries in a comprehencive analysis of large scale spatial-genetic patterns and trends of genetic variation in Europe were also lacking. The goal of this thesis was to provide information on wolf population structure and processes in Europe with a particular emphasis on Estonia and Latvia, including the wolf-dog hybridization.

Hybridization between grey wolf and domestic dog was ascertained in Estonia (for the first time) and Latvia using a combined analysis of maternal, paternal and biparental genetic markers. Six hybrid individuals from Estonia and two from Latvia were initially detected from their atypical morphological traits and their hybrid status was subsequently confirmed using genetic analysis. Analysis of mtDNA showed that the two hybrids from Latvia represented a very rare case of hybridization – the first record from Europe – between a female dog and a male wolf.

Population genetic analysis demonstrated that the relatively small wolf population shared between Estonia and Latvia is represented by four genetic groups. While three of the four genetic groups were geographically well defined, being either Estonian or Latvian based, one of the groups was distributed widely in Estonia and Latvia. The spatially explicit DResD analysis provided clear evidence of spatial variation of genetic divergence: (a) at the smallest spatial scale (20–80 km) several blending areas of different groups appeared with relatively high genetic distance between otherwise geographically closely positioned individuals; (b): at the medium scale (80–140 km) a putative territory of an expanding pack was detected in south-west Estonia, coinciding with one of the core areas of group D (c): at the largest spatial scale (140–250 km) a large area in the north-eastern part of Estonia was identified as a migration corridor.

To describe large-scale trends and patterns of genetic variation in European wolf populations, we conducted a meta-analysis based on the results of previous microsatellite studies and included also new data, covering all 19 European countries for which wolf genetic information is available: Norway, Sweden, Finland, Estonia, Latvia, Lithuania, Poland, Czech Republic, Slovakia, Germany, Belarus, Russia, Italy, Croatia, Bulgaria, Bosnia and Herzegovina, Greece, Spain and Portugal. We compared different indices of genetic diversity in wolf populations and found a significant spatial trend in heterozygosity across Europe from south-west (lowest genetic diversity) to north-east (highest). The range of spatial autocorrelation calculated on the basis of three characteristics of genetic

diversity was 650−850 km, suggesting that the genetic diversity of a given wolf population can be influenced by populations up to 850 km away.

Various human-related factors are undoubtedly the main source of threats to wolf populations in Europe: the majority of populations face similar common threats such as overharvesting (including poaching), low public acceptance, conflicts due to livestock depredation, habitat destruction, barriers to gene flow and interactions with dogs leading to possible hybridization. For the long-term survival and favourable conservation status of European wolves there is a need to increase the overall population size and favour wolf dispersal and connectivity among and within populations. For science-based wolf conservation and management at regional and Europe-wide scales it was suggested (1) to manage wolf populations according to biological units, which requires additional genetic analysis covering all wolf populations in Europe to define the exact number and spatial distribution of populations; (2) to increase scientific knowledge and inform stakeholders and the general public, there is a need to establish a European Union Wolf Scientific Committee and a European Union Reference Laboratory of Wolf Studies.

SUMMARY IN ESTONIAN

Hundi (*Canis lupus*) populatsioonid Eestis ja Euroopas: geneetiline mitmekesisus, populatsiooni struktuur ja -protsessid ning hübridiseerimine koertega

Teadmised hundi populatsioonigeneetikast ja huntide hübridiseerumisest koertega on olulised nii kohaliku populatsiooni tasandil kui ka liigi tõhusa kaitse- ja majandamisotsuse tegemiseks kogu Euroopas. Euroopa eri riikides ja piirkondades paiknevate hundipopulatsioonide geneetikat ning huntide-koerte hübridiseerumist käsitlevaid uuringuid oli enne doktoritööga alustamist avaldatud mitmeid, kuid andmed Balti populatsiooni, eriti Eesti ja Läti huntide kohta, puudusid. Samuti oli selge vajadus läbi viia uuring, mis analüüsiks suuremastaabilisi ruumilis-geneetilise variatsiooni mustreid ja trende üle Euroopa. Selle väitekirja eesmärk oli täita eelpool nimetatud tühimikud ja anda teavet hundi populatsioonigeneetika kohta Euroopas, rõhuga Eesti ja Läti hundipopulatsioonidele.

Kasutades kombineeritud analüüsi, mis kaasab nii emaliini, isaliini kui ka biparentaalseid geneetilisi markereid, tuvastati Eestis (esmakordselt) ja Lätis hübridiseerumine hundi ja koera vahel. Kuus hübriidset isendit Eestist ja kaks Lätist tuvastati algselt nende ebatavaliste morfoloogiliste tunnuste tõttu ning nende hübriidne staatus kinnitati hiljem geneetilise analüüsiga. MtDNA analüüs näitas, et kaks hübriidi Lätist esindasid väga haruldast, Euroopas esmakordselt tuvastatud hübridisatsioonijuhtumit – emase koera ja isase hundi vahel.

Eestit ja Lätit hõlmav hundi populatsioonigeneetiline analüüs näitas, et hoolimata asjaolust, et Eesti ja Läti hundipopulatsioonid on väiksearvulised, esineb neli geneetilist rühma. Kolm neljast geneetilisest rühmast jaotusid riigiti, kaks gruppi olid Eesti- ja üks Läti-keskne, ent neljas grupp esines laialdaselt üle Eesti ja Läti. Uudne DResD analüüs tuvastas selge ruumilise erinevuse geneetilises lahknemises: (a) väikseimal ruumilisel skaalal (20–80 km) identifitseeriti mitmed erinevate gruppide segunemisalad, millel paiknesid geograafiliselt üksteisele lähedased, kuid geneetiliselt suhteliselt kauged isendid; (b) keskmise suurusega (80–140 km) skaalal tuvastati oletatav territooriumit laiendav kari Edela-Eestis ja (c) suurimal ruumilisel skaalal (140–250 km) määratleti suur ala Kirde-Eestis kui hundi rändekoridor.

Selleks, et kirjeldada laiaulatuslikke trende ja geneetilise variatsiooni mustreid Euroopa hundipopulatsioonides, viisime läbi meta-analüüsi, kasutades nii olemasolevaid mikrosatelliitmarkeritel põhinenud uuringuid, kui ka uusi andmeid, hõlmates kokku 19 Euroopa riiki: Norra, Rootsi, Soome, Eesti, Läti, Leedu, Poola, Tšehhi, Slovakkia, Saksamaa, Valgevene, Venemaa, Itaalia, Horvaatia, Bulgaaria, Bosnia ja Hertsegoviina, Kreeka, Hispaania ja Portugal. Analüüsiti hundipopulatsioonide erinevaid geneetilise mitmekesisuse indekseid ja leiti Euroopa-ülene ruumiline heterotsügootsuse trend suunaga edelast kirdesse – madalaim geneetiline mitmekesisus esines Euroopa edelaosas ja kõrgeim kirdeosas. Ruumilise autokorrelatsiooni vahemikuks, mis arvutati kolme geneetilise

mitmekesisuse tunnuse abil, saadi 650–850 km, mis viitab sellele, et konkreetse hundipopulatsiooni geneetilist mitmekesisust võivad mõjutada hundipopulatsioonid, mis asuvad kuni 850 km kaugusel.

Peamised hunte ohustavad tegurid Euroopas on inimtegevusega seotud, mistõttu seisab enamik Euroopa hundipopulatsioonidest silmitsi sarnaste ohtudega: küttimine (sealhulgas salaküttimine), inimeste madal sallivus hundi suhtes, konfliktid kariloomade murdmise tõttu, elupaikade hävimine ning võimalik hübridiseerumine koertega. Liigi pikaaegseks säilimiseks ja soodsa seisundi saavutamiseks Euroopas on vaja suurendada populatsioonide üldist arvukust, soodustada loomade levikut ja populatsioonide-siseseid ja -vahelisi seoseid. Hundi teaduspõhiseks kaitseks ja majandamiseks nii piirkondlikel kui ka üle-Euroopalisel skaalal on soovitatav hallata hundipopulatsioone kui bioloogilisi üksusi, mis nõuab kõiki Euroopa hundipopulatsioone hõlmavaid täiendavaid geneetilisi analüüse, et teha kindlaks populatsioonide täpne arv, ruumiline jaotus, geenisiirde ulatused ning hübridiseerumise sageduse koertega.

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